IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Anticipated Classification of this Application:
Class ____ Subclass ____

Prior Application:
Examiner Gambel, P.
Group Art Unit 1644

Attorney's Docket No. 48879-B/JPW/JSG

A

HONORABLE ASSISTANT COMMISSIONER FOR PATENTS
Washington, D.C. 20231

jc377 U.S. PTO
09/23/99

September 23, 1999

JC572 U.S. PTO
09/404076
09/23/99

S I R:

This is a request for filing a X CONTINUATION ____ DIVISIONAL ____ CONTINUATION-IN-PART application under X 37 C.F.R. § 1.53(b) ____ 37 C.F.R. §1.62[1], of pending prior application Serial No. 08/763,669 filed on December 11, 1996 of Robert E. Canfield, Steven Birken, John O'Connor and Galina Kovalevskaka for

Inventor(s)

ANTIBODIES SPECIFIC FOR HLH BETA CORE FRAGMENT AND USES THEREOF

Title of Invention

1. ____ Enclosed is a copy of the prior application, as originally filed and an affidavit or declaration verifying it as a true copy.

2. X A verified statement to establish small entity status under 37 C.F.R. §1.9 and 1.27

   ____ is enclosed.

   X was filed in the prior application and such status is still proper and desired (37 C.F.R. §1.28(a)); and a copy is enclosed.

3. ____ The filing fee is calculated as follows:

CLAIMS AS FILED, LESS ANY CLAIMS CANCELLED BY AMENDMENT

	NUMBER FILED		NUMBER EXTRA*		RATE SMALL ENTITY	RATE OTHER ENTITY		FEE SMALL ENTITY	FEE OTHER ENTITY
Total Claims	2 - 20	=	0	X	$ 9	$ 18	=	$ 0	$ —
Independent Claims	1 - 3	=	0	X	$ 39	$ 78	=	$ 0	$ —
Multiple Dependent Claims Presented:	____ Yes X No				$ 130	$ 260	=	$ 0	$ —
					BASIC FEE			$ 380	$ 760
					TOTAL FEE			$ 380	$ —

*If the difference in Col. 1 is less than zero, enter "0" in Col. 2.

[1] filing an application pursuant to this section expressly abandons the parent application.

4. __X__ The Commissioner is hereby authorized to charge payment of the following fees associated with this application or credit any overpayment to Deposit Account No._______.

__X__ Any additional filing fees required under 37 C.F.R. §1.16.

__X__ Any patent application processing fees under 37 C.F.R. §1.17.

_____ The issue fees set forth in 37 C.F.R. §1.18 at or before mailing of the Notice of Allowance, pursuant to 37 C.F.R. §1.311(b).

5. __X__ Three copies of this sheet are enclosed.

6. __X__ A check in the amount of $__380.00__ is enclosed.

7. __X__ Cancel claims __5 - 26__.

8. _____ Amend the specification by inserting before the first line the sentence: --This is a ___continuation ___division of application Serial No.__________, filed __________.--

9. __X__ _10_ Sheet(s) of ___ informal _X_ formal drawing(s) is/are enclosed.

10. _____ Transfer the drawings from the prior application to this application and abandon said prior application as of the filing date accorded this application. A duplicate copy of this sheet is enclosed for filing in the prior application file.

11. _____ Priority of application No. __________ filed on ________________ in ______________ (country) is claimed under 37 U.S.C. §119.

_____ The certified copy of the priority application has been filed in prior application Serial No. ____________, filed ____________.

12. __X__ The prior application is assigned of record to The Trustees of Columbia University in the City of New York.

13. __X__ A preliminary amendment is enclosed.

14. __X__ The power of attorney in the prior application is to:

John P. White (Reg. No. 28,678); Christopher C. Dunham (Reg. No. 22.031); Norman H. Zivin (Reg. No. 25.385); Jay H Maioli (Reg. No. 27,213); William E. Pelton (Reg. No. 25.702); Robert D. Katz (Reg. No 30.141), Peter J. Phillips (Reg. No. 29,691) Wendy E. Miller (Reg. No. 35,615); Richard S. Milner (Reg. No 33.970), Albert Wai-Kit Chan (Reg. No. 36.479); Robert T. Maldonado (Reg. No. 38.232); Paul Teng (Reg No. 40.837); Gary J. Gershik (Reg. No. 39.992), Richard F Jaworski (Reg. No. 33.515); Elizabeth M. Wieckowski (Reg. No. 42.226); and Pedro C. Fernandez (Reg. No. 41.741)

(a) X The power appears in the original papers in the prior application.

(b) ___ Since the power does not appear in the original papers, a copy of the power in the prior application is enclosed.

(c) X Address all future communications to: (May only be completed by applicant, or attorney or agent of record.)

John P. White
Cooper & Dunham LLP
1185 Avenue of the Americas
New York, New York 10036

15. X Also enclosed Express Mail Certificate of Mailing No. EL 278 885 968 US dated September 16, 1999

16. X I hereby verify that the attached papers are a true copy of prior application Serial No. 08/763,669 as originally filed on December 11, 1996.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statement and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

September 23, 1999
Date

Signature John P. White, Reg. No. 28,678

___ INVENTOR(S)
___ ASSIGNEE OF COMPLETE INTEREST
X ATTORNEY OR AGENT OF RECORD
___ FILED UNDER 37 C.F.R. §1.34(a)

Address of Signator:

Cooper & Dunham LLP
1185 Avenue of the Americas
New York, New York 10036

Applicant or Patentee: Robert E. Canfield, et al. Attorney's
Serial or Patent No.: Not Yet Known Docket No: 48879-A/JPW/A
Filed or Issued: Herewith
Title of Invention or Patent: ANTIBODIES SPECIFIC FOR HLH BETA CORE FRAGMENT USES THEREOF

VERIFIED STATEMENT (DECLARATION) CLAIMING
SMALL ENTITY STATUS UNDER 37 C.F.R. §1.9(f)
AND §1.27(d) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Organization: The Trustees of Columbia University in the City of New York

Address of Organization: West 116th Street and Broadway
New York, New York 10027, U.S.A.

TYPE OF ORGANIZATION:

X UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
___ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE 26 U.S.C. §§501(a) and 501(c)(3)
___ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
NAME OF STATE: ___
CITATION OF STATUTE: ___
___ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE 26 U.S.C. §§501(a) and 501(c)(3) IF LOCATED IN THE UNITED STATES OF AMERICA
___ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
NAME OF STATE: ___
CITATION OF STATUTE: ___

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 C.F.R. §1.9(e)* for purposes of paying reduced fees under 35 U.S.C. §41(a) and 41(b), with regard to the invention entitled

by inventor(s) Robert E. Canfield, et al.

described in:

___ the specification filed herewith
X application serial no. Not Yet Known filed Herewith
___ patent no. ___ issued ___

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive each individual, concern, or organization known to have rights to the invention is listed below[a] and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 C.F.R. §1.9(d)* or a nonprofit organization under 37 C.F.R. 1.9(e)*

[a]NOTE: Separate verified statements are required from each person, concern, or organization having rights to the invention averring to their status as small entities. 37 C.F.R. §1.27.

Name: N/A
Address:

___ Individual ___ Small Business Concern ___ Nonprofit Organization

*See Reverse

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. 37 C.F.R. §1.28(b)*.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing: Mr. Jack M. Granowitz
Title In Organization: Executive Director, Columbia Innovation Enterprise
Address: 500 West 120th Street, Engineering Terrace - Suite 363, Mail Code 2206
New York, New York 10027
Signature: Jack M. Granowitz
Date Of Signature: 12/10/96

*See Reverse

Dkt. 48879-B/JPW/JSG

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Robert E. Canfield, et al.

Serial No.: Not Yet Known (Continuation application of U.S. Serial No. 08/763,669, filed December 11, 1996)

Filed : Herewith

For : ANTIBODIES SPECIFIC FOR HLH BETA CORE FRAGMENT AND USES THEREOF

1185 Avenue of the Americas
New York, New York 10036
September 23, 1999

Assistant Commissioner for Patents
Washington, D.C. 20231
Box: Patent Applications

SIR:

PRELIMINARY AMENDMENT

Please amend the subject application as follows:

In the specification:

On page 1, line 4 of the specification after the words, "This application", and before the words, "claims priority of", please insert the following:

--is a continuation of U.S. Serial No. 08/763,669, filed December 11, 1996 which--.

On page 1, line 6 of the specification delete the word --which-- and insert the following words:

--these applications are--.

<u>In the claims</u>:

Please cancel original claims 2-3 and 5-26 without prejudice to applicant's right to pursue the subject matter of these claims in a future continuation or divisional application.

Please amend claims 1 and 4 under the provisions of 37 C.F.R. §1.121(b) by deleting the bracketed word or words and inserting the underlined word or words as follows:

--1. (Amended) An antibody which specifically binds to <u>human luteinizing hormone beta core fragment, (</u>hLHβcf<u>)</u> without cross-reacting with <u>human luteinizing hormone (</u>hLH<u>), human luteinizing hormone free beta subunit (</u>hLHβ<u>) or human chorionic gonadotropin beta core fragment (</u>hCGβcf<u>)</u>.--

--4. (Amended) An <u>anti</u>-hLHβcf antibody which completitively inhibits the binding of the antibody of claim 1.--

REMARKS

Claims 1-26 were pending in the subject application. Applicants have hereinabove canceled claims 2-3 and 5-26 without prejudice.

Applicants have hereinabove amended claims 1 and 4. Applicants maintain that support for the amended claim 1 may be found *inter alia* on page 24, Table 2, wherein the affinity to hLHβcf and cross-reactivitry with hLH, hLHβ or hCGβcf of several antibodies are summarized. Applicants maintain that support for the amended claim 4 may be found *inter alia* on page 25, lines 28-34 to page 26, lines 1-15, wherein a summary of simultaneous interactions of two antibodies with hLHβcf indicates a competition for the same antigen at the same site exists. Applicants maintain that the amendments to the claims do not involve any issue of new matter and

respectfully request entry of these amendments. Accordingly claims 1 and 4 will be pending upon entry of this amendment.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone at the number provided below.

No fee is deemed necessary in connection with the filing of this Preliminary Amendment. If any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,

John P. White
Registration No. 28,678
Attorney for Applicants
Cooper & Dunham, LLP
1185 Avenue of the Americas
New York, New York 10036
(212) 278-0400

Application
for
United States Letters Patent

To all whom it may concern:

Be it known that Robert E. Canfield, Steven Birken, John O'Connor and Galina Kovalevskaya *have invented certain new and useful improvements in* ANTIBODIES SPECIFIC FOR HLH BETA CORE FRAGMENT AND USES THEREOF

of which the following is a full, clear and exact description.

ANTIBODIES SPECIFIC FOR HLH BETA CORE FRAGMENT AND USES THEREOF

This application claims priority of U.S. provisional application No. 08/008,502, filed December 11, 1995, the content of which is hereby incorporated into this application by reference.

The invention disclosed herein was made with United States Government support under National Institute of Health Grants, HD 15454 and ES-07589. Accordingly, the United States Government has certain rights in this invention.

Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citations for these references may be found at the end of this application, preceding the claims.

Background of the Invention

Recently, applicants isolated an hLH beta core fragment (hLHβcf) from human pituitaries. This molecule is homologous to the hCG beta core fragment (hCGβcf), which may be a marker of normal pregnancy, Down syndrome, and certain cancers. Applicants now report antibodies to the hLHβcf, four of which have been applied in sensitive immunoradiometric assays for urinary measurements. One of the antibodies recognizes an epitope on the hLHβcf, which is not present on the hCGβcf, hLH, or hLHβ. This specific hLHβcf antibody acts cooperatively with other newly-developed antibodies reported here to produce an assay with a sensitivity of 1 fmol/ml of hLHβcf. The specificity of these new IRMA systems will make it possible to measure the

hLHβcf in urine in the presence of hLH, hLH beta, or the hCGβcf. Although the hLHβcf used to develop specific antibodies was purified from pituitaries, the assays developed recognize this metabolite in urine. Measurements of heterodimeric hLH as compared to hLHβcf in the urine of cycling women indicated that the concentration of hLHβcf rose as high as 6-7 times the concentration of hLH starting a day after the midcycle surge. The new measuring systems allow the precise quantitation of this hLH metabolite in urine.

Understanding of the metabolites of the gonadotropins excreted into urine may help to distinguish between healthy and abnormal physiological states. For example, the hCG β core fragment (hCGβcf) is present at high levels in the urine of normal pregnant women (Kato et al., 1988) but, also, occurs abnormally in the urine of nonpregnant patients with a variety of malignancies (O'Connor et al., 1988, Cole et al., 1988a,1988b,1990). Applicants and others have observed a beta core fragment of hLH (hLHβcf) in the urine of normally cycling women shortly after the hLH midcycle surge (Neven et al., 1993) and in the urine of postmenopausal women (Iles et al., 1992). Both the hCG and hLH fragments have analogous structures (Birken et al., 1993) but, it has not been possible to measure one of the fragments in the presence of the other. For example, the utility of the hCGβcf molecule as a marker of malignancies in postmenopausal women has been compromised by the cross-reactions of antibodies elicited to the hCGβcf with a molecule of similar structure and size (presumably the homologous fragment of hLH) excreted by normal postmenopausal women in their urine. Consequently, the high threshold measurement compromised the ability of hCGβcf to serve as a cancer marker in this important patient population. Applicants had earlier suggested the

hypothesis that, if it were possible to distinguish an hLHβcf from an hCGβcf, a preponderance of the former might be indicative of the normal state while a major mole fraction of the hCG fragment may be associated with malignancy (Birken et al., 1993). Immunological analysis of the hLHβcf in normal cycling women, as compared with infertile patients, may identify a metabolic marker associated with an abnormal state (i.e.an ovulatory cycles, polycystic ovarian disease). For these reasons, applicants have developed a series of antibodies to the hLHβcf , which was isolated from a pituitary extract but, as reported here, can also be used to measure such a molecule in urine.

Although antibodies to the hCGβcf could be used to extract the hLH-associated core materials from normal postmenopausal women, it was difficult to generate sufficient material to even characterize the structure of the molecule present in urine. Instead, applicants were able to successfully isolate an hLHβcf from human pituitary extracts(Birken et al., 1993). Using this material, applicants now report the development and characterization of immunometric measurement systems to quantitate the pituitary hLHb core fragment in urine. These assays will now make it possible to evaluate the metabolism of hLH in both pre and postmenopausal women and to possibly distinguish between normal and abnormal physiological states.

Summary of the Invention

This invention provides an antibody which specifically binds to hLHβcf without cross-reacting with hLH, hLHβ or hCGβcf. In an embodiment, the monoclonal antibody is designated B505. In a further embodiment, the hybridoma cell line producing the monoclonal antibody B 505 is designated ATCC Accession No.HB-12000. This invention also provides hLHβcf antibody which competitively inhibits the binding of the monoclonal antibody B505.

This invention provides a method for determining the amount of hLHβcf in a sample comprising steps of:(a) contacting at least one capturing antibody selected from a group consisting of B503, B504 and B509 with a solid matrix under conditions permitting binding of capturing antibody with the solid matrix; (b) contacting the bound matrix with the sample under conditions permitting binding of the antigen present in the sample with the capturing antibody;(c) separating the bound matrix and the sample;(d) contacting the separated bound matrix with an antibody which specifically binds to hLHβcf without cross reacting with hLH, hLHβ or hCGβcf; and (e) determining the amount of bound antibody on the bound matrix, thereby determining the amount of hLHβcf in the sample. In an embodiment, the antibody is B505.

In performing the above method, the separation of the bound matrix and the sample in step (c) may be carried out by:(i) removing of the sample from the matrix, and (ii)washing the bound matrix with an appropriate buffer. Alternatively, they may be separated by other methods known in the art.

This invention also provides a method of detecting ovulation in a female subject comprising:(a) obtaining samples from

the female subject; and (b) determining the amount of hLHβcf in the samples, the presence of a peak of hLHβcf indicating the occurrence of ovulation.

This invention further provides the above method, wherein step (b) comprising: (i) contacting the sample with an antibody which specifically binds to hLHβcf without cross-reacting with hLH, hLHβ or hCGβcf under conditions permitting formation of complex between the antibody and hLHβcf; and (ii) determining the amount of the complex, thereby determining the amount of hLHβcf in the samples. This invention further provides the above method, wherein the antibody is labelled with a detectable marker.

This invention provides a method for reducing the amount of hLHβcf in a sample comprising steps of: (a) contacting the sample with an antibody which specifically binds to hLHβcf without cross-reacting with hLH, hLHβ or hCGβcf under conditions permitting formation of complex between the antibody and hLHβcf; and (b) removing the complex formed, thereby the amount of hLHβcf in the sample.

This invention also provides the above method, wherein the removing step comprising: (i) contacting the complex with protein A under conditions permitting formation of protein A with an antibody; and (ii) removing the complex formed, thereby the amount of hLHβcf in the sample.

In an embodiment of this method, the complex is contacted with a secondary antibody under conditions permitting binding of the secondary antibody to the first antibody prior to step (i). In a separate embodiment of this method, the antibody is linked to a solid matrix.

This invention further provides samples with reduced amount

of hLHβcf produced by the above-described methods.

Brief description of the Figures

Figure 1 Antibody dilution curves for the 9 hybridoma supernatants with ^{125}I-hLHβcf in liquid phase RIA. Dilution of cell supernatant appears on the X-axis while the total counts of tracer bound appears on the Y-axis.

Figure 2 Liquid phase competition curves of the binding of ^{125}I-hLHβcf with unlabeled hLHβcf, hLH, hCGβcf is shown for the four antibodies: B509, B504, B503, B505. Panel B shows the most specific antibody, B505, which does not appear to bind any hCGβcf nor hLH in liquid phase assays.

Figure 3 Competitive curves of the binding of mABs in solution with mABs immobilized on the plate for binding to ^{125}I-hLHβcf in solid phase RIA. Panel C shows the enhancement of binding of tracer when either antibodies B503 or B504 is added to B505 immobilized on the plate. This enhancement is due to the cooperativity in formation of "a circular complex" (Ehrlich et al., 1982) and has led to a two-site assay of extraordinary sensitivity with an extended measurement range.

Figure 4 The hormonal profiles of two ovulatory menstrual cycles from normal women (patient #1 and #2). All values have been normalized to creatinine. Panels A in both subjects show values for intact hLH, hLHβ and hLHβcf in urine. Panels B provide data on two urinary steroid metabolites, estrone-3-glucuronide and pregnanediol-3-glucuronide. Note that in both subjects the concentrations of hLHβcf substantially exceed that of the intact

hLH and hLHβ and that its maximum excretion appears to lag that of hLH and hLHβ by one day.

Figure 5 HPLC elution positions of the pituitary and urinary hLHβcf. The open circles denote the elution position of hLHβcf derived from the pituitary. The closed circles denote the elution position of hLHβcf - partially purified from urine. The difference in elution position denotes a structural difference (probably carbohydrate differences) between the two forms. The column separates molecules on the basis of hydrophobicity. Both the urinary molecule and the pituitary derived molecule exhibit immunoreactivity with B505 as well as B503, B504, and B509.

Figure 6 Study of rechromatography of the pituitary hLHβcf on reverse phase HPLC in order to calculate true cross-reactivity of pituitary hLHβcf in the assay which has been used for measurement of urinary hCGβcf (B210-B108). The concentration of pituitary hLHβcf as well as the concentration of hCGβcf were measured in each of the same column fractions of a single separation. The concentration of pituitary hLHβcf was determined by B505-B503 assay and appears on the left Y-axis while the concentration measured by the hCGβcf assay appears on the right axis as determined by the B210-B108 assay using urinary hCG fragment standard. The latter assay is presumed to measure true cross-reactivity of pure pituitary hLHβcf within fractions 40-45 while 37-39 may represent the slight contamination with pituitary hCGβ which appears prior to pituitary hLHβcf in

this system (Hoermann et al., 1995). Note that the left axis of panel A is pmole/ml while the right axis, representing the hCGβcf, is in fmol/ml showing that the cross reaction of the hCGβcf (B210-B108) assay with the hCGβcf is very low as is the contamination with the pituitary hCGβcf. Lower panel B shows the position of urinary hCGβcf on this column system which presumably elutes in a similar fashion to authentic pituitary hCGβcf (Hoermann et al., 1995).

Figure 7 HLH and hLHβcf in serum and urine of the same patient. The blood levels of intact hLH (open circles) and hLHβcf (closed circles) are illustrated in the upper panel. It indicates that there is an insignificant amount of the hLHβcf detected in the blood. The lower panel illustrates the urinary values for hLH and hLHβcf in the urine for the same days of collection. The surge of hLH(day 0) and the surge of hLHβcf (1-2 days later) are detected in urine, but the peak of hLHβcf lags that of the intact hLH by 1-2 day, suggesting that the origin of urinary hLHβcf is the peripheral or renal metabolic processing of intact hLH.

Detailed Description of the Invention

This invention provides an antibody which specifically binds to hLHβcf without cross-reacting with hLH, hLHβ or hCGβcf. In an embodiment, the monoclonal antibody is designated B505. In a further embodiment, the hybridoma cell line producing the monoclonal antibody B 505 is designated ATCC Accession No.HB-12000.

This hybridoma cell was deposited on December 11, 1995 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.S. under the provision of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. This hybridoma has been accorded with ATCC Accession No. 12000.

This invention also provides hLHβcf antibody which competitively inhibits the binding of the monoclonal antibody B505.

This invention provides a method for determining the amount of hLHβcf or hLHβcf-related molecule in a sample comprising steps of: (a) contacting the sample with an antibody which specifically binds to hLHβcf without cross-reacting with hLH, hLHβ or hCGβcf under condition permitting formation of a complex between the antibody and hLHβcf; and (b) determining the amount of complexes formed, thereby determining the amount of hLHβcf or hLHβcf-related molecule in the sample. In an embodiment, the antibody is produced by the hybridoma cell line accorded with ATCC Accession No.12000. In another embodiment, the antibody is labelled with a detectable marker. In a further embodiment, the antibody is radioactively labelled.

As the methodology of radioimmunoassay (RIA) is well known in this art, an ordinary skilled artisan can easily use this methodology for determining the amount of hLHβcf or hLHβcf-related molecule in a sample using the disclosed antibodies.

This invention provides a method for determining the amount of hLHβcf or hLHβcf-related molecule in a sample comprising steps of:(a) contacting at least one capturing antibody selected from a group consisting of B503, B504 and B509 with a solid matrix under conditions permitting binding of capturing antibody with the solid matrix; (b) contacting the bound matrix with the sample under conditions permitting binding of the antigen present in the sample with the capturing antibody;(c) separating the bound matrix and the sample;(d) contacting the separated bound matrix with an antibody which specifically binds to hLHβcf without cross reacting with hLH, hLHβ or hCGβcf; and(e) determining the amount of bound antibody on the bound matrix, thereby determining the amount of hLHβcf or hLHβcf-related molecule in the sample. In an embodiment, the antibody is B505. Methods for determining the amount of antibody bound to an antigen are well-known in the art. For example, the detecting or the secondary antibody may carry a detectable marker. A standard curve may be generated using known amounts of the tested antigen and the amount of signal generated by the marker.

This invention also provides monoclonal antibodies, B503, 504 and 509. This invention also provides hybridoma cell lines producing the monoclonal antibody B503, 504 and 509. These hybridoma cell lines were deposited on December 11, 1995 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.S. under the provision of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes

of Patent Procedure. These hybridoma have been accorded with ATCC Accession Nos.11999, 12001 and 12002 respectively.

In performing the above method, the separation of the bound matrix and the sample in step (c) may be carried out by: (i) removing of the sample from the matrix, and (ii)washing the bound matrix with an appropriate buffer. Alternatively, they may be separated by other methods known in the art.

This invention also provide methods for determining the amount of hLHβcf or hLHβcf-related molecule in a sample comprising steps of: (a) contacting a capturing antibody which specifically binds to hLHβcf without cross-reacting with hLH, hLHβ or hCGβcf with a solid matrix under conditions permitting binding of the antibody with the solid matrix; (b) contacting the bound matrix with the sample under conditions permitting binding of the antigen present in the sample with the bound capturing antibody; (c) separating the bound matrix and the sample; (d) contacting the separated bound matrix with at least one detecting antibody selected from a group consisting of B503, B504 and B509 under conditions permitting binding of antibody and antigen in the sample; and (e) determining the amount of bound antibody on the bound matrix, thereby determining the amount of hLHβcf or hLHβcf-related molecule in the sample.

In an embodiment, the antibody which specifically binds to hLHβcf without cross-reacting with hLH, hLHβ or hCGβcf is B505. In a further embodiment, the antibody is labelled with a detectable marker. In a still further embodiment, the detectable marker is a radioactive isotope, enzyme, dye or biotin. In a further embodiment, the radioactive isotope is I^{125}.

This invention also provides a method of detecting ovulation

in a female subject comprising:(a) obtaining samples from the female subject; and (b) determining the amount of hLHβcf or hLHβcf-related molecule in the samples, the presence of a peak of hLHβcf or related molecule indicating the occurrence of ovulation.

This invention further provides the above method, wherein step (b) comprising: (i) contacting the sample with an antibody which specifically binds to hLHβcf without cross-reacting with hLH, hLHβ or hCGβcf under conditions permitting formation of complex between the antibody and hLHβcf; and (ii) determining the amount of the complex, thereby determining the amount of hLHβcf or related molecule in the samples.

This invention further provides the above-method, wherein the antibody is labelled with a detectable marker.

In an embodiment, the monoclonal antibodies of this invention are labelled with a detectable marker, for example, a radioactive isotope, enzyme, dye or biotin. In a further embodiment, the radioactive isotope is I^{125}.

In an embodiment of the above described method, the sample tested is a urine sample. In a separate embodiment, the sample is a blood sample.

This invention provides a method for reducing the amount of hLHβcf or related molecule in a sample comprising steps of:(a) contacting the sample with an antibody which specifically binds to hLHβcf without cross-reacting with hLH, hLHβ or hCGβcf under conditions permitting formation of complex between the antibody and hLHβcf; and (b) removing the complex formed, thereby the amount of hLHβcf or hLHβcf related molecule in the sample.

This invention provides the above method, wherein the removing step comprising: (i) contacting the complex with protein A under conditions permitting formation of protein A with an antibody; and (ii) removing the complex formed, thereby the amount of hLHβcf or hLHβcf related molecule in the sample.

In an embodiment of this method, the complex is contacted with a secondary antibody under conditions permitting binding of the secondary antibody to the first antibody prior to step (i). In a separate embodiment of this method, the antibody is linked to a solid matrix.

This invention further provides samples with reduced amount of hLHβcf produced by the above-described methods.

As stated herein, samples include but not limited to urine sample and blood samples.

It is clear that all the methods described in this invention are applicable to hLHβcf-related molecules. Such molecules are defined as molecules capable of being recognized by the antibody which specifically binds to hLHβcf without cross-reacting with hLH, hLHβ or hCGβcf. Specifically, the hLHβcf-related molecules may be recognized by B505.

This invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

Materials and Methods

Preparation of hLHβcf

The extraction of the hLHβcf from human pituitary extracts was reported earlier(Birken et al., 1993). Applicants prepared approximately 700μg of hLHβcf from 8g of starting human pituitary glycoprotein extract.

Other hormones

HLH was obtained from two different sources. One preparation of hLH was a gift from Dr. Anne Stockell Hartree (Hartree, 1975). This preparation of hLH was completely intact by amino acid sequence analysis. A second preparation of hLH (AFP 8270B), as well as one of hLH beta (AFP 3282B), used in these studies were obtained from the National Pituitary Agency. Which preparation was used in various studies is indicated within the text. The isolation of hCGβcf was described earlier (Birken, et al., 1988). ^{125}I- hLH was obtained from Diagnostics Products Corporation.

Iodination of hLHβcf and hCGβcf

HLHβcf and hCGβcf were iodinated using Iodogen (Pierce Chemical Co., Rockford, Ill.) according to manufacturer's instructions.

Purification and iodination of monoclonal antibodies.

Immunoglobulins were purified from ascites by the Protein A Monoclonal Antibody Purification System (Bio-Rad, Richmond, CA.). The protein concentration of pure antibodies was determined by amino acid analysis. Purification of mABs was checked by a PAGE in the presence of SDS according to Laemmli (Laemmli, 1953). Pure antibodies were labeled with ^{125}I by chloramine T-method (Hunter and Greenwood, 1962). Not less then 70% of the radioactivity was able to bind specifically hLHβcf.

Immunization of mice

Balb/c mice were immunized twice subcutaneously with 4-6 μg of hLHβcf per each animal in complete (first immunization) or incomplete (second immunization) Freund's adjuvant. The second immunization was carried out on day 14 after the first immunization. On days 21 and 28 the mice were immunized intraperitoneally (ip) with 4μg of antigen per animal. On the day 35 blood was taken and sera were tested for antibodies. Mice with high antibody response were boosted with 6 μg hLHβcf iv and after 3 days used for fusion.

Cell fusion

Spleen cells from immunized mice were fused with cells of myeloma line X63-Ag8.653 3 days after the booster injection according to the method of Kohler and Milstein (Kohler and Milstein, 1975). The splenocyte to myeloma cell ratio was 4:1 or 5:1. Polyethylene glycol 4000 (Sigma, St. Louis, MO.) was used as fusing reagent. After fusion, cells were distributed in 6 microtitration plates on mouse peritoneal feeder cells and cultured for one week in HAT-selection RPMI 1640 or DMEM media containing 20% FCS. One half of the medium was replaced every 3 days. One week after fusion, HAT-medium was changed for HT. On day 12-14 post fusion, culture supernatants (100 ml) from the wells with cell clones were screened for the presence of antibodies to hLHβcf using liquid phase RIA. Positive selected cells were cloned at least two times by limiting dilutions on mouse peritoneal feeder cells. Subclones were injected intra peritoneally into Balb/c mice ($0.5\text{-}1\times10^6$ cells/mouse) and the ascites produced were used as source of mABs. Hybridoma cells were stored in liquid nitrogen in FCS containing 10% DMSO.

<u>Screening of primary clones</u>

Primary screening was carried out in liquid phase RIA with ^{125}I-hLHβcf. The liquid phase RIA procedure was described earlier (Birken et al., 1980). In brief, the binding buffer consisted of PBS supplemented with 0.1% BSA and 0.02% sodium azide. 150 ml solution containing 30,000-40,000 cpm ^{125}I-hLHβcf was added to 100 ml culture supernatant diluted 2.5:1 with PBS. 50 ml of 8% normal mouse serum was also added. This solution was incubated for 1h at 37 C and after that overnight at 4 C. Then 500 ml of a 2.5% goat anti-mouse serum was added and mixture was incubated for 1h at 37 C and for 2h at room temperature. The precipitate containing bound radioactive hLHβcf was separated by centrifugation and counted in a gamma counter. Supernatants of positive clones were tested in the same kind of assay to check cross-reactivity with ^{125}I-hCGβcf and ^{125}I-hLH. Immune serum as a positive control was used.

<u>Competitive liquid phase RIA</u>

Competitive liquid phase radioimmunoassays were conducted as follows: Cell supernatants were used in those dilutions at which approximately 40% of maximum antibody binding occurred in the absence of unlabeled hormones. The following reagents were added to each 12 x 75mm polystyrene tube: 100 ml diluted supernatant, 30,000-40,000 cpm of ^{125}I-hLHβcf in 300 ml binding buffer (PBS, pH 7.2 with 0.1% BSA), 100ml competitor solution and 100 ml 8% normal mouse serum. After incubation for 1h at 37 C and overnight at 4 C, 1 ml 2.5% goat anti-mouse serum was added as in the primary screening. The cross reactivity of different competitors was calculated by the PC version of the program Allfit written by DeLean et al. (De Lean et. al., 1992). Likewise, affinity constants were calculated by homologous competitive displacement assays using the PC version of the program Ligand by Munson (Munson and Rodbard, 1980).

Competitive solid phase RIA

Each antibody was adsorbed onto the wells (100 ml per well) of microtiter plates (Immulon II, Dynatech, Chantilly, VA.) by incubating a solution of the antibody (B503-2μg/ml, B504-1μg/ml, B505-5μg/ml, B509-5μg/ml) in 0.2 M bicarbonate, pH 9.6 overnight at 4 C. The coating antibody solution was aspirated, the plates were washed with PBS and blocked with 2% solution of BSA in PBS for 3h at room temperature. The blocking solution was removed, the plates were washed with PBS and 100 ml of binding mixture was added to each well. The binding mixture, which contained ^{125}I-hLHβcf and dilutions of antibodies in PBS with 0.1% bovine gamma globulin, was preincubated at 37 C for 1 h. After an incubation for 2 h at room temperature and overnight at 4 C the solution was aspirated, the plates were washed with PBS and bound radioactivity was counted. Results were presented as percentages of ^{125}I-hLHβcf binding in the absence of competitor.

IRMA

Applicants' methodology for the construction and validation of Immunometric assays has been fully described (O'Connor et al., 1988). Briefly, the specificity of the antibody pairs and their capacity for simultaneous binding to antigen are determined as follows. The analytes tested for potential cross reaction with the hLHβcf monoclonal antibodies included hCGβcf, hLH (AFP 8270B), hLH free β subunit (AFP 3282B), intact hCG (CR 127) and hCG free β subunit (CR129). The degree of cross reaction was anticipated from a knowledge of antibody specificity in liquid phase RIA. The range of the β core LH standards was 3.9 to 1000 fmol/ml. The range of cross reactants encompassed 39 to 278000 fmol/ml, depending on the analyte.

The capture antibody (marked by a single asterisk in Table

2) was adsorbed onto the wells of microtiter plates by incubating a 20 μg/ml solution of the antibody in coating buffer (0.2 M bicarbonate, pH 9.5) overnight at 4 C. The coating antibody solution was aspirated, the plates washed (wash solution 0.9% NaCl, 0.05% Tween 20) and blocked with a 1% solution of BSA in water. Following incubation with the BSA solution (minimum 3 hours at room temperature) the blocking solution was removed, the wells again washed and 200 ml/well of the appropriate hLHβcf standards or potential cross-reacting molecules were added in phosphate buffer B (0.05M phosphate with 0.1% bovine gamma globulin and 0.1% NaN_3). After overnight incubation at 4 C, the plates were again aspirated and washed. The 200ml (50,000 cpm)of appropriate ^{125}I-labeled detection antibody (listed with double asterisks in Table 2) was added to the wells which were again incubated for 24h at 4C. The tracer was aspirated, the plates washed with water, the individual well placed in glass tubes and the radioactivity determined in a Packard Cobra gamma counter. Doses were determined by interpolation from a smoothed spline transformation of the data points.

In addition to hLHβcf assays, three other assays, described earlier, were used for hLH and hLHβ (Krichevsky et. al., 1994) and for the hCGβcf (Krichevsky et al., 1991).

For the assay of urinary hLH and its metabolic forms, the following antibody pairs were employed: For intact hLH, B406*-A201**; for the hLH free beta subunit, B408*-B409**; and for the hLHβcf B505*-B503**. Prior to assay, the urines are thawed, the pH is adjusted with 1.0M Tris (pH 9.5), 50μl/ml urine, and aliquoted (200μl/well) into 96 well microtiter plates which had been previously coated with capture antibody and blocked with BSA. A serially diluted standard curve of the appropriate analyte (intact hLH, hLH

free beta subunit or hLH beta core fragment) is added in buffer B to the wells and the plate is incubated overnight at 4C. The assay is performed from that point identically to that described for antibody characterization.

Steroid glucuronide enzyme immunoassay

The solid phase ELISAs for estrone 3-glucuronide and pregnanediol 3-glucuronide were performed with reagents provided by Drs. Bill Lasley and George Stobenfield of the University of California, Davis. The assay has been fully described previously (Krichevsky et al., 1994).

Isotyping of mABs

Isotypes of mABs were determined using Mouse Monoclonal Sub-isotyping Kit (HyClone, Logan, Utah) according to the manufacturer's instruction except that the plate was coated with hLHβcf (0.1mg/well) instead of rabbit anti-mouse immunoglobulins.

Experimental Result

In order to choose antibodies specific to the hLHβcf, applicants selected for high affinity binding to the hLHβcf, which was the immunogen, and also, for very low or no binding to hCGβcf and to hLH and free hLHβ. The extensive homology among these three hormone fragments as well as the scarcity of the hLHβcf prompted us to employ radiolabeled molecules for initial screening of the supernates of cells during the clonal selection process. Splenocytes from animals displaying high serum titers to the radiolabeled hLHβcf were fused with high efficiency (75-85%). Three fusions were successful in producing a large number of cell lines which bound radiolabeled hLHβcf. A total of 112 positive clones was produced. Each well supernate was ranked in terms of binding specificity by assigning the

supernate from wells which bound the highest amounts of radiolabeled hLHβcf as 100%. The same procedure was used to set the maximal binding of radiolabeled of hLH and hCGβcf. Assuming that each well supernate contained about the same quantity of antibody, the relative percentage of binding of each radiolabeled protein was calculated. Examination of the data indicated that 60% of positive clones (clones with cell supernates that bound hLHβcf) recognized all three radiolabeled proteins, 12% bound both hLHβcf and hCGβcf, 8% recognized hLHβcf and hLH, and 20% of the clones appeared fairly specific to the hLHβcf. Those clones which demonstrated the best growing characteristics were subcloned at least twice and sufficient cell supernatants of each clone was produced for further characterization studies. Titration binding curves of supernatants from clones of interest were performed in liquid phase RIA using ^{125}I-hLHβcf as a tracer (Figure 1). This study permits rapid comparisons of the relative antibody affinity of each of the clones (Heyningen et al., 1983). It was assumed that the concentration of antibodies in each supernatant varied only slightly. The titration study shows that mABs B509, B503 and B504 have the highest affinities. Although antibody B505 has a lower affinity than these other antibodies, it has the best specificity for the hLHβcf and, thus, it was also selected for further study.

Four antibodies to the hLHβcf, B505, B509, B504, and B503 were characterized for relative specificities and sensitivities in a series of competition curves using radiolabeled hLHβcf and unlabeled hLHβcf, hCGβcf and hLH as competitors. The results of these studies are summarized in Figure 2 and Table 1.

Table 1. Characteristics of mABs to hLHβcf

Antibody	Isotype	K_a, M^{-1}, (cv, %)	ED+/-SE, hLHβcf, pmole/ml	ED+/-SE, hLH, pmole/ml	ED+/-SE, hCGβcf, pmole/ml	Cross-reactivity**, hLH, %	Cross-reactivity** hCGβcf, %
B505	G1	$3.01x10^8$ (86)	6.49+/-0.326	>>80	>>320	nd	nd
B509	G1	$1.37x10^{10}$ (9)	0.228+/-0.0089	6.135+/-0.72	>140	3.72	<0.16
B504	G1	$2.06x10^{10}$ (10)	0.205+/-0.011	0.157+/-0.011	1.385+/-0.088	130	14
B503	G2a	$1.31x10^{10}$ (11)	0.335+/-0.0097	0.953+/-0.035	0.414+/-0.013	35	80.9

*ED-concentration of hormones needed to inhibit 50% of 125-iodo-hLHβcf binding to various mABs in liquid phase RIA; **-was determined in liquid phase RIA; SE-standard error; nd-not determined

These antibodies were characterized (Table 1) in terms of their isotype, affinity constants, and cross-reactivity. Figure 2, which presents liquid phase competition studies, shows that all four of these antibodies are different in their relative binding characteristics. Antibody B509 is slightly cross-reactive with hLH and hCGβcf (Fig 2A); Antibody B504 binds hLH and hLHβcf approximately equally (Fig 2C); Antibody B503 binds all three competitors in a very similar fashion (Fig 2D). Antibody B505 binds hLHβcf quite specifically (Fig 2B). Although liquid phase cross-reactivities are not paralleled precisely in the two-site format solid phase assay, the liquid phase data indicates that these four antibodies are different and may have different binding sites making them amenable to two-site assay development. The quantitative analysis of sensitivities and cross reactivities for these four antibodies are summarized in Table 1. Three antibodies (B503, B504 and B509) displayed high affinities in the 10^{10} M^{-1} range. Antibody B505 was in the range of 10^{8} M^{-1}. The cross-reactivity of antibody B505 with the hCGβcf and with hLH were too low to measure.

Table 2 details the characteristics of two-site IRMAs developed using the new antibodies described in this report. The four monoclonal antibodies described in this report functioned in combination with each other to produce excellent immunometric assays for hLH-beta core fragment. Analytes tested for cross reactivity in these systems included hCG beta core fragment, hLH, hLH free beta subunit, hCG, and hCG free beta subunit.

Table 2. Characterization of immunoradiometric assays for hLHβcf

			Cross-reactivity with analyte					
Assay	Bmax, %	LDD, fmol/ml	hLHβcf, %	hCGβcf, %	hLH, %	hLHβ, %	hCG, %	hCGb, %
B505*-B503**	**83**	**1.3**	**100**	**0.1**	**1.1**	**1.3**	**0.2**	**1.4**
B505*-B504**	71	<<4	100	0.05	1.3	<<0.05	0.43	2.6
B505*-B509**	39	4	100	0	0	0	0	0
B509*-B503**	86	<4	100	6	6	1	0.3	3
B509*-B504**	90	<<4	100	5.8	6.5	1.1	0.4	3.1
B509*-B505**	3	125	<1	<1	<1	<1	<1	<1
B201*-B108**	50	0.7	2	100	<1	<1	1	<1

*-Antibody immobilized on the solid phase, **-antibody labeled with ^{125}I, LDD-lowest detectable dose, Bmax-max binding of total count

The most useful assays were provided by employing either B509 or B505 as capture and B503 or B504 for detection. In all of the above combinations, a sensitivity of less than four fmoles/ml was realized (sensitivity defined as NSB+3SD). The assay which provided the best combination of sensitivity and specificity proved to be the B505 capture, B503 detection system. The sensitivity of this configuration was about one fmole/ml and the cross reaction with all of the tested analytes was under 2%. Cross-reaction with the hCG beta core fragment was less than 0.1% while cross-reaction with hLH was about 1%. However, even better specificity is afforded by the B505*-B509** combination, in which it was not possible to detect any cross-reactivity with the other analytes over the range tested. This configuration has the disadvantages of both decreased sensitivity (4 fmol/ml vs about 1 fmol/ml for B505*-B503**) and a diminished B-max relative to the other assays, probably reflecting partial overlap of the two epitopes. Nevertheless, in those instances where extreme sensitivity is not required, but in which any cross-reacting analytes are present, then the B505*-B509** configuration is certainly an acceptable alternative. The last row of Table 2 indicates the cross-reactivity of applicants' previously developed two-site immunoassay to the hCGβcf (B210*-B108**) with pituitary hLHβcf to be approximately 2%.

A detailed analysis of the simultaneous interactions of two antibodies with the hLHβcf was conducted to distinguish those antibodies which cannot bind simultaneously from those that bind at the same time. Enhanced simultaneous binding is especially desirable. The study of the interactions of the four hLHβcf antibodies was accomplished using iodinated hLHβcf, one immobilized solid phase antibody and one liquid phase antibody (Gomez and Retegui, 1994). These findings are illustrated in Fig 3. The results of these studies

indicated that antibodies B503 and B504 competed for antigen and were clearly directed to the same antibody binding site. With immobilized B505, all three other anti-hLHβcf antibodies demonstrated binding synergism or cooperativity. The binding of labeled hLHβcf to immobilized B505 more than doubles in the presence of B503 and B504 (Fig 3C). The effect was most pronounced with mABs B504 and B503, less so for B509, which appears to share an overlapping site with B505. Antibodies B505 and B509 bind to different sites on the hLHβcf than do B503 and B504. No other antibody combination other than those with immobilized B505 display binding cooperativity. Cooperativity between B505 and B503 detection has permitted the construction of a highly sensitive (1 fmol/ml) immunometric assay for hLHβcf having a wide dynamic range (0-1000 fmol/ml).

MAB B505 performs only marginally or not at all as a detection antibody when labeled with ^{125}I. This inhibition applies whether the iodination is performed by either Chloramine T or the Iodogen techniques. This suggests that perhaps a tyrosine(s) in or near the binding site is affected by iodine substitution.

The potential clinical utility of these assays is illustrated by the menstrual cycle profiles of 7 normally ovulating women two of whom are presented in Figure 4. In these cycles the peak excretion of hLHβcf lags that of the intact hLH at least by one day. The values for hLHβcf in these subjects exceed those of hLH and hLHβ (both of which peaked the same day) by 6-7 fold (Fig 4) . One patient exhibited a rise in hLHβ immunoreactivity one day prior to the hLH surge and this patient appears in Figure 4. Measurement of the urinary steroid metabolites estrone-3-glucuronide and pregnanediol-3-glucuronide confirmed that the ovulation had occurred in these cycles (Fig 4, Panel B).

There appears to be a basal pulsatile concentration of the hLHβcf in the urine.

Experimental Discussion

Although a variety of hLH antibodies have been reported in the literature during the past several years (Krichevsky et al., 1994, Alonso-Whipple et al., 1988,Odell and Griffin, 1987), this is the first report of antibodies and two-site assays specific to the hLHβcf. In fact, applicants have only recently confirmed the existence of the hLHβcf by structural studies of this core material isolated from a pituitary extract (Birken et al., 1993). These new antibodies and the IRMA systems described in this report should provide important reagents to determine the pattern of excretion of this metabolite into urine. A molecule of the size and immunochemical properties of this metabolite appears to be present during the normal ovulatory cycle after the hLH surge (Neven et al., 1993) and is present in postmenopausal women (Iles et al., 1992). Those investigators used antibodies developed to the hCGβcf which they hypothesized to cross-react with a putative hLHβcf in urine. However, without antibodies individually specific for only one of the β core metabolites, it is not possible to distinguish hLHβcf from hCGβcf. The pattern of occurrence of such gonadotropin metabolites may provide important clinical information related to the health of a patient. For example, although the hCGβcf has been identified as a marker molecule associated with a variety of malignancies (O'Connor et al., 1988; Cole et al., 1988a, 1988b, 1990; O'Connor et al., 1994), its value as such a marker in postmenopausal women has been limited by the presence of an immunochemically cross-reacting molecule of similar size (Iles et al., 1992). This molecule is likely to be derived from hLH and is probably the hLHβcf. Development of the specific two-site assays described in

this report should make it possible to accurately measure the concentration of hLHβcf in the presence of the hCGβcf as well as high levels of hLH in urine. These assays may have a direct application for studies of markers related to menopause, the ovulatory cycle, as well as to distinguish normal postmenopausal women from those with cancers.

Since purified hLHβcf was scarce, antigen-conserving techniques were used to select the desired antibodies. Although applicants wished to measure hLHβ metabolites in urine, applicants decided to pursue development of antibodies to a pituitary form of the hLHβcf since applicants had already isolated this material in a highly purified form. Applicants had not been able to isolate hCG-core fragment cross-reactive material directly from postmenopausal urine (Birken et al., 1993) but assumed it was a molecule derived from hLH based on the studies of Iles (Iles et al., 1992) and applicants' own work. The supply of pituitary hLHβcf was quite limited since its yield was only about 100μg/g of crude pituitary extract. There were a number of considerations in selection of antibodies to this molecule. First, it was a low abundance protein within the pituitary extract. Therefore, the screening of antibody-producing cell supernates was done exclusively by radiolabeled protein because of the low supply of hLHβcf and the need to conserve protein for competition experiments later on. Secondly, since the structures of the hCG and hLHβcfs were very similar, it was likely to prove difficult to select antibodies which could clearly distinguish between the two molecules. Third, it was also necessary to select against binding to hLH and hLHβ since both are present in postmenopausal urine, as well as at the mid-cycle hLH surge in ovulating women, and their cross-reactions would complicate measurement of the hLHβcf. Fourth, it was necessary to select antibodies of medium to high affinity in

order to be able to measure low levels of the hLHβcf in urine. Fifth, it was also necessary to select a set of antibodies which could be used in two-site measurement of the hLHβcf. The latter requirement made it necessary to develop a variety of antibodies to the hLHβcf.

The strategy used to select the diverse antibodies needed for development of the appropriate two-site assay was screening candidate antibody-secreting cells with three radiolabeled tracers: hLHβcf, hCGβcf and hLH. The resulting titration patterns from three fusions permitted selection of four cell lines secreting the appropriate antibodies. Liquid phase assay studies indicated that B505 was specific for hLHβcf (i.e. displayed no detectable cross-reaction with either the hCGβcf or hLH at the concentrations used). Antibody B509 was nearly equally specific for the hLHβcf versus the hCG fragment but displayed binding (3.72% in competitive liquid phase RIA) with hLH (Table 1). Two other antibodies bound all three proteins and proved excellent candidates for the second antibody in a two-site assay. Indeed, a two-site assay using B505 as capture and B503 as detection antibody was developed and displayed approximately 1% cross-reaction with hLH and hLHβ and 0.1% cross-reaction with the hCGβcf. Examination of the Table 2 indicates that this is the most satisfactory combination of antibodies for use in postmenopausal urine measurements, as well as measurements during the ovulatory cycle. Using liquid phase assays, it was found that the sensitivity of antibody B505 was only 7% (by ED50 calculations) that of B509 for the hLHβcf (Table 1). Yet, when two-site assays were developed separately for both antibodies, it was found that both exhibited the same sensitivity of less than 4 fmol/ml. This detection level sensitivity has proved to be more than adequate for the clinical measurements which applicants intend to perform. The solid-phase format resulted in a

significant increase in antibody sensitivity in this case. The reason for the increase in sensitivity of B505 in solid phase assays is due to the cooperativity effect between B505 and B503 or B504. This effect arises from the formation of "a circular complex" of antibodies binding sites when the antibodies are positioned at appropriate distances from each other on the surface of a ligand, and is known to result in a much higher affinity than that of either antibody alone (Ehrlich et al., 1982). The affinity increases without any compromise of the excellent specificity of B505. This increase in affinity is very clearly shown in Table 2 and in Figure 3.

The finding that the hLHβcf displays a unique epitope, which is not present on the hCGβcf nor on the hLH beta subunit, was surprising since the two fragments are very similar in primary sequence. The difference presumably lies within a variation of the structures of the two core fragments. Although the hLHβcf was isolated from a pituitary extract, the resultant antibodies detect this material in the urine of a normal cycling woman coincident with and then peaking a day or more after the hLH peak. This delay may result from metabolic processing of hLH within a peripheral compartment followed by the delayed release of fragments into urine. Studies by conducted by Dr. Nisula and colleagues by injection of hCG, hCGβ subunit and hCGβ core fragment into human volunteers as well as into animals showed that only 8% of injected hCG beta core fragment appears in the urine while 22% of injected hCG and 0.7% of hCGβ subunit enter the urine (Wehmann et al., 1989, Wehman and Nisula, 1981). The remainder of the molecules are taken-up by liver, ovary and kidney tissues and disposed of by routes other than urine. This group showed that after infusion of the hCG beta core fragment, its excretion into urine persists for as long as 7 days and they hypothesize uptake by renal parenchymal

cells and slow re-excretion into urine (Wehmann et al., 1989). Such an uptake and re-excretion mechanism may explain the delay in appearance of the hLHβcf in urine after the hLH surge. Although the uptake and processing of hCG into hCGβcf is thought to occur within the kidney, it is not yet known where hLHβcf may be taken up and processed since the molecule is present within the pituitary and may be present in the circulation at higher levels than those very low levels observed for the hCGβcf. Further insight into the origin and clearance rate of the hLHβcf await optimization of serum and plasma assays and the ensuing clinical studies.

Iles et al. (Iles et al., 1992), Neven et al. (Neven et al., 1993) as well as the applicants (unpublished observations) have observed a periovulatory signal in the hCGβcf assay when menstrual cycle hormone profiles are examined. Immunological evidence has indicated that this signal is due in part to cross-reaction with an hLH associated molecule, but that conclusion was based on assays whose cross-reaction with hLHβcf was unknown. The appearance of substantial quantities of immunoassayable hLHβcf, as assessed by applicants' specific hLHβcf assay, in the hormone profile of normally cycling women, suggest this is in fact the case. The basal pulsatile concentration of the hLHβcf during the follicular phase in these cycling women probably reflects the metabolic processing of the normal circulating pulsatile hLH in blood during this time period. Conclusive evidence of the nature of these molecules awaits their isolation and structural determination. Applicants do not know as yet if the structure of this hLHβ core fragment present a mid-cycle in urine is identical to that isolated from pituitary although they share at least one unique epitope. Likewise, the structure of the hLHβ core in postmenopausal urine also remains to be defined. However, applicants report here a

quantitative immunoassay for urinary hLHβcf using pituitary hLHβcf as standard allowing expression in molar units. Applicants have found that applicants' current hCGβcf assay cross-react with the hLHβcf 2% on a molar basis.

There are numerous reports in the literature that hLH exists as a variety of isoforms in the circulation and that many monoclonal antibodies fail to recognize some of these forms and produce erroneous measurement results (Petterson et al., 1991,1992; Stanton et al., 1993; Martin-Du-Pan et al., 1994). In fact, some hLH serum assays indicated the absence of hLH in a patient while other assays show normal levels (Petterson et al., 1991). An analogous measurement problem is probably even more serious in urine since more degraded hLH molecules are likely to be present. As is the case for hCG, hLH appears to be metabolized to a beta subunit fragment of similar structure to the hCG beta core fragment upon passage into urine.

An additional potential application for these novel measuring systems may be in cancer diagnostics as described in the introduction. The hCGβcf has proven useful as a marker of gynecological cancer (Cole et al. 1988a, 1988b, 1990; O'Connor et al., 1988). Its usefulness is compromised by the simultaneous presence of an immunologically interfering substance in urine, especially postmenopausal women (Iles et al., 1992). It may be possible to use one of the hLHβcf antibodies as a scavenger for the hLH cross-reacting materials to reduce the threshold background so that the hCGβcf assays may be more useful for cancer detection and monitoring of cancer therapy.

The availability of these new hLHβcf antibodies now makes possible the conduct of clinical studies of this hLH metabolite in the urine of patients. These new immunometric

assays provide the tools to study the relationship of the presence of this metabolite as compared to the analogous metabolite of hCG as indicative of health or disease. The extremely sensitive IRMA system for measurement of hLHβcf will be applied to the study of this excreted hLH metabolite in the urine of normal cycling women, infertility patients and as a possible marker of the onset of menopause.

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What is claimed is:

1. An antibody which specifically binds to hLHβcf without cross-reacting with hLH, hLHβ or hCGβcf.

2. The monoclonal antibody of claim 1 designated B505.

3. A hybridoma cell line producing the monoclonal antibody of claim 2 (ATCC Accession No.12000).

4. An hLHβcf antibody which competitively inhibits the binding of the monoclonal antibody of claim 1.

5. A method for determining the amount of hLHβcf or hLHβcf-related molecule in a sample comprising steps of:
   (a) contacting the sample with an antibody which specifically binds to hLHβcf without cross-reacting with hLH, hLHβ or hCGβcf under condition permitting formation of a complex between the antibody and hLHβcf; and
   (b) determining the amount of complexes formed, thereby determining the amount of hLHβcf or hLHβcf-related molecule in the sample.

6. The method of claim 5, wherein the antibody is produced by the hybridoma cell line accorded with ATCC Accession No.12000.

7. A method for determining the amount of hLHβcf or hLHβcf-related molecule in a sample comprising steps of:

   (a) contacting at least one capturing antibody selected from a group consisting of B503, B504

and B509 with a solid matrix under conditions permitting binding of capturing antibody with the solid matrix;

(b) contacting the bound matrix with the sample under conditions permitting binding of the antigen present in the sample with the capturing antibody;

(c) separating the bound matrix and the sample;

(d) contacting the separated bound matrix with an antibody which specifically binds to hLHβcf without cross reacting with hLH, hLHβ or hCGβcf under conditions permitting binding of antibody and antigen in the sample; and

(e) determining the amount of bound antibody on the bound matrix, thereby determining the amount of hLHβcf or hLHβcf-related molecule in the sample.

8. A method of claim 7, wherein the antibody is B505.

9. A method of claim 7, wherein the step (c) comprising:

(i) removing of the sample from the matrix, and

(ii) washing the bound matrix with an appropriate buffer.

10. A method for determining the amount of hLHβcf or hLHβcf-related molecule in a sample comprising steps of:

(a) contacting a capturing antibody which

specifically binds to hLHβcf without cross-reacting with hLH, hLHβ or hCGβcf with a solid matrix under conditions permitting binding of the antibody with the solid matrix;

(b) contacting the bound matrix with the sample under conditions permitting binding of the antigen present in the sample with the bound capturing antibody;

(c) separating the bound matrix and the sample;

(d) contacting the separated bound matrix with at least one detecting antibody selected from a group consisting of B503, B504 and B509 under conditions permitting binding of antibody and antigen in the sample; and

(e) determining the amount of bound antibody on the bound matrix, thereby determining the amount of hLHβcf or hLHβcf-related molecule in the sample.

11. A method of claim 10, wherein the antibody which specifically binds to hLHβcf without cross-reacting with hLH, hLHβ or hCGβcf is B505.

12. A method of claim 10, wherein the antibody is B503.

13. A method of claim 5, 7 or 10, wherein the antibody is labelled with a detectable marker.

14. A method of claim 13, wherein the detectable marker is a radioactive isotope, enzyme, dye or biotin.

15. A method of claim 14, wherein the radioactive isotope

is I^{125}.

16. A method of detecting ovulation in a female subject comprising:

    (a) obtaining samples from the female subject; and

    (b) determining the amount of hLHβcf or hLHβcf-related molecule in the samples, the presence of a peak of hLHβcf indicating the occurrence of ovulation.

17. A method of claim 16, wherein step (b) comprising:

    (i) contacting the sample with an antibody which specifically binds to hLHβcf without cross-reacting with hLH, hLHβ or hCGβcf under conditions permitting formation of complex between the antibody and hLHβcf; and

    (ii) determining the amount of the complex, thereby determining the amount of hLHβcf or hLHβcf-related molecule in the samples.

18. A method of claim 17, wherein the antibody is labelled with a detectable marker.

19. A method of claim 18, wherein the detectable marker is a radioactive isotope, enzyme, dye or biotin.

20. A method of claim 19, wherein the radioactive isotope is I^{125}.

21. A method for reducing the amount of hLHβcf or hLHβcf-related molecule in a sample comprising steps of:

(a) contacting the sample with an antibody which specifically binds to hLHβcf without cross-reacting with hLH, hLHβ or hCGβcf under conditions permitting formation of complex between the antibody and hLHβcf; and

(b) removing the complex formed, thereby the amount of hLHβcf or hLHβcf-related molecule in the sample.

22. A method of claim 21, wherein the removing step comprising:

(i) contacting the complex with protein A under conditions permitting formation of protein A with an antibody; and

(ii) removing the complex formed, thereby removing the amount of hLHβcf or hLHβcf-related molecule in the sample.

23. A method of claim 22, further comprising contacting the complex with a secondary antibody under conditions permitting binding of this secondary antibody with the first antibody prior to step (i).

24. A method of claim 21, wherein the antibody is linked to a solid matrix.

25. The sample with reduced amount of hLHβcf or hLHβcf-related molecule produced by the method of claim 21.

26. A method of claim 5, 7, 10, 16 or 21, wherein the sample is a urine sample or a blood sample.

ANTIBODIES SPECIFIC FOR HLH BETA CORE FRAGMENT AND USES THEREOF

ABSTRACT OF THE DISCLOSURE

This invention provides an antibody which specifically binds to hLHβcf without cross-reacting with hLH, hLHβ or hCGβcf. In an embodiment, the monoclonal antibody is designated B505. In a further embodiment, the hybridoma cell line producing the monoclonal antibody B 505 is designated ATCC Accession No.12000. This invention provides different uses of the antibodies. Finally, this invention provides a method for determining the amount of hLHβcf or hLHβcf-related molecule in a sample.

FIGURE 1

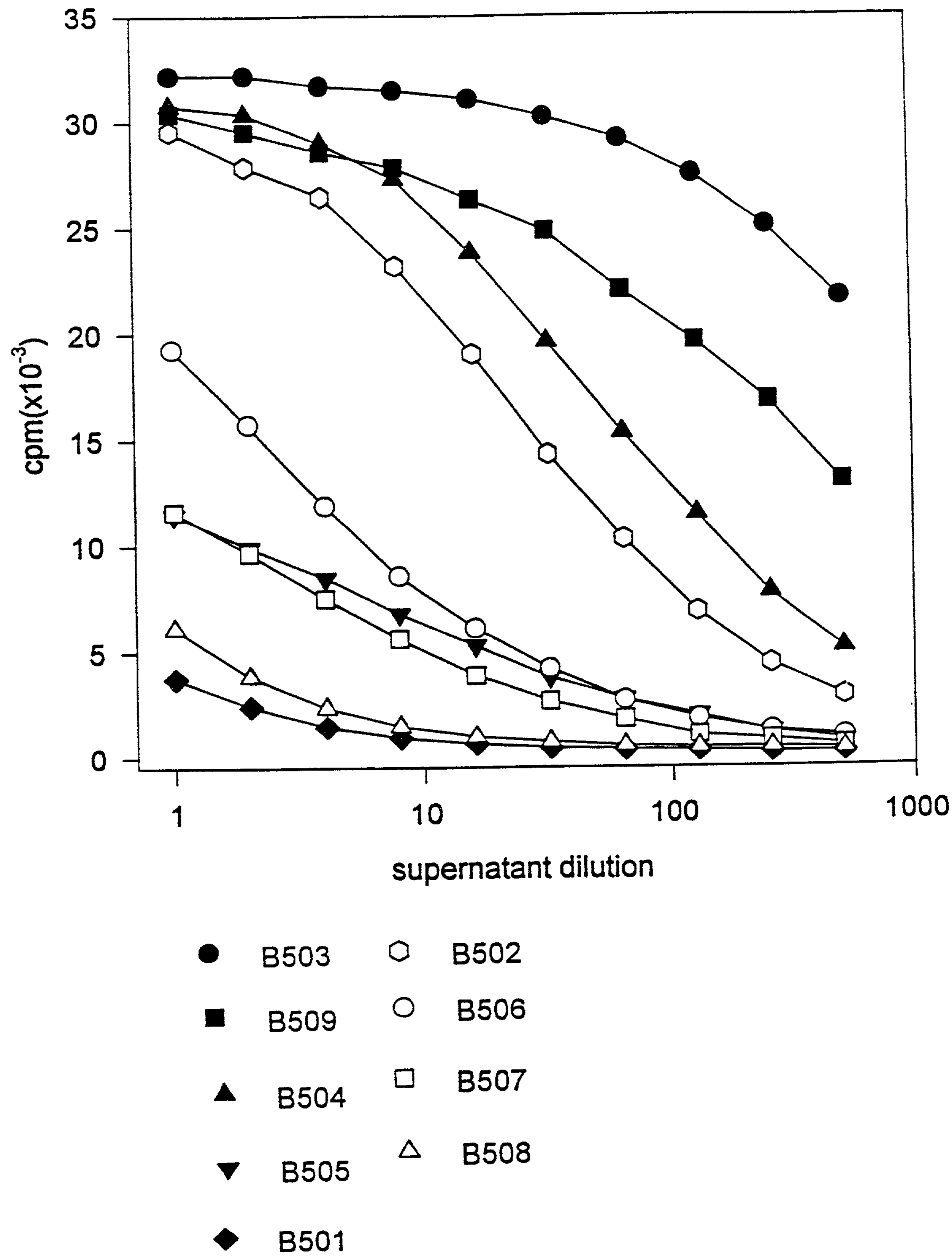

35
30
25
20
15
10
5
0
cpm(x10^{-3})
1
10
100
1000
supernatant dilution
B503
B502
B509
B506
B504
B507
B505
B508
B501

FIGURE 2A

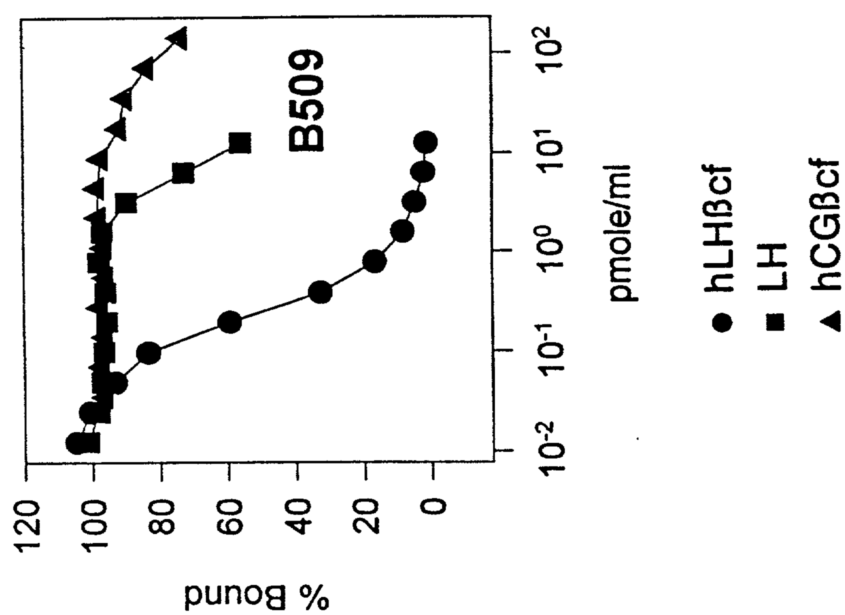


FIGURE 2B

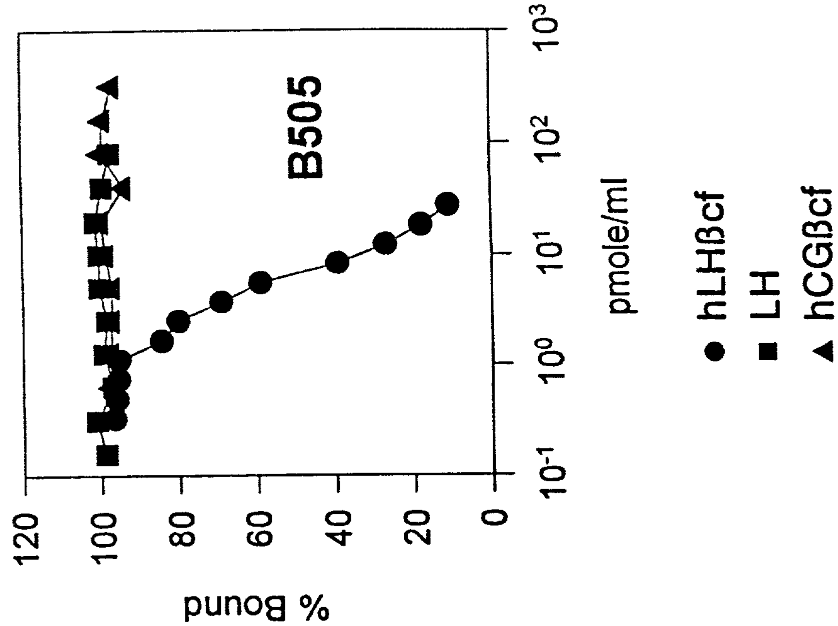


FIGURE 2C

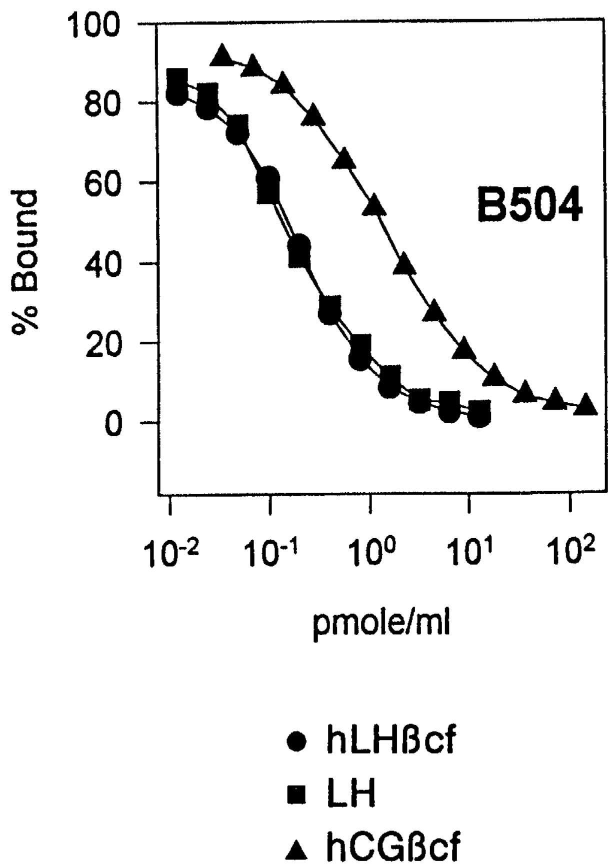


FIGURE 2D

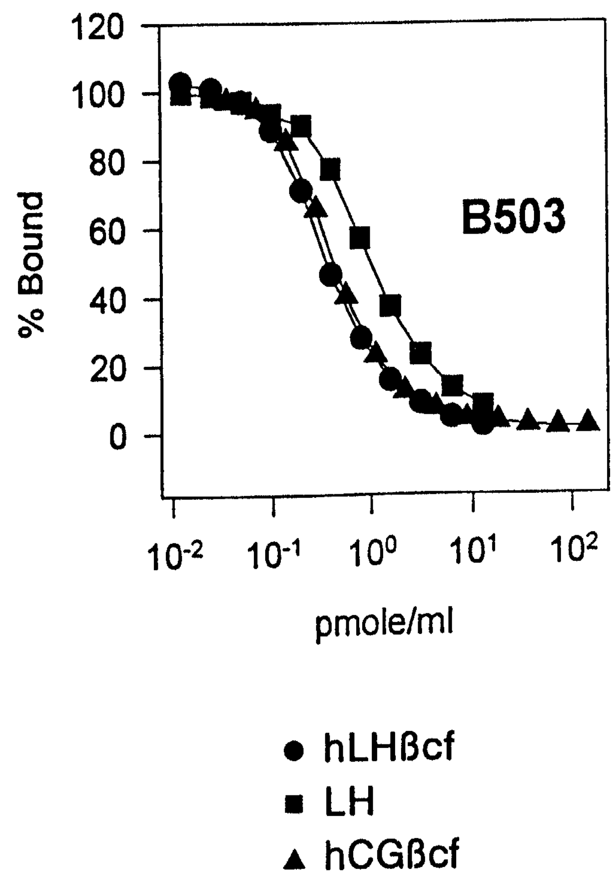


FIGURE 3A

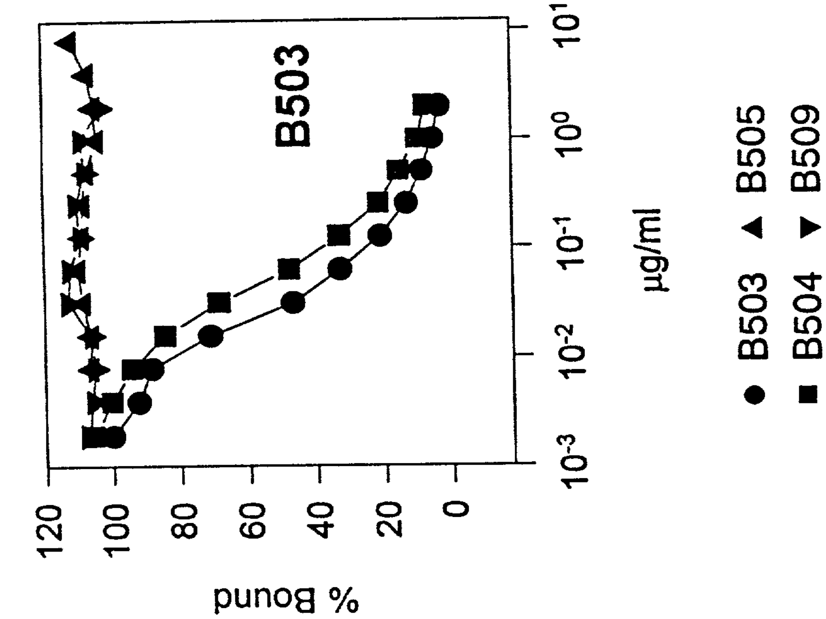


FIGURE 3B

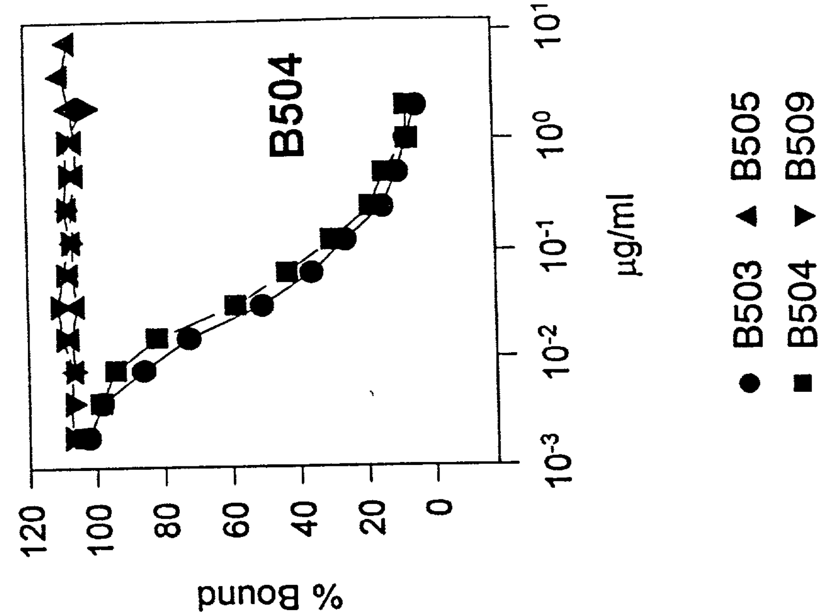


FIGURE 3C

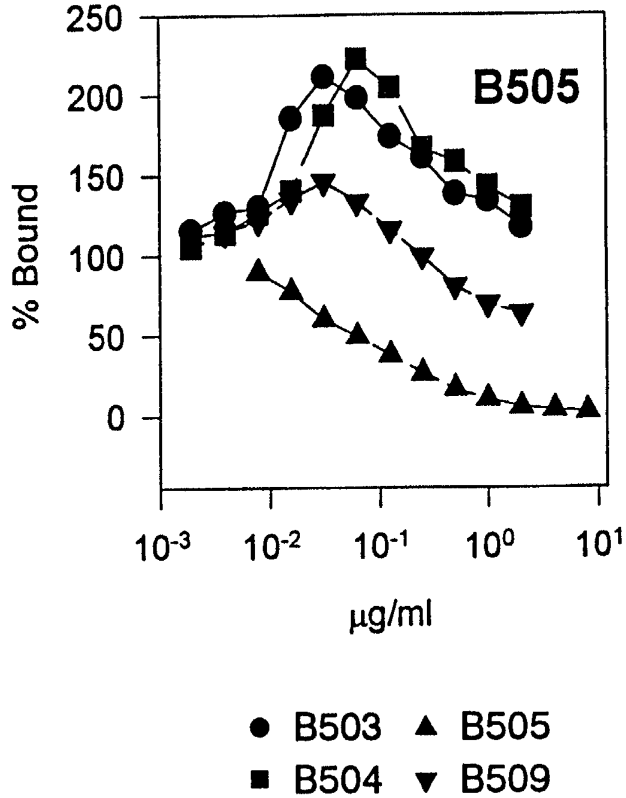
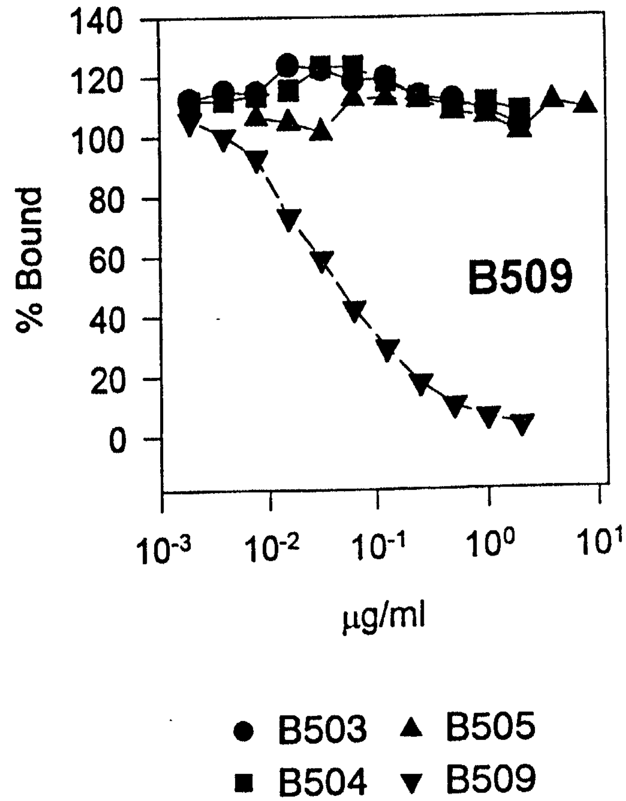


FIGURE 3D



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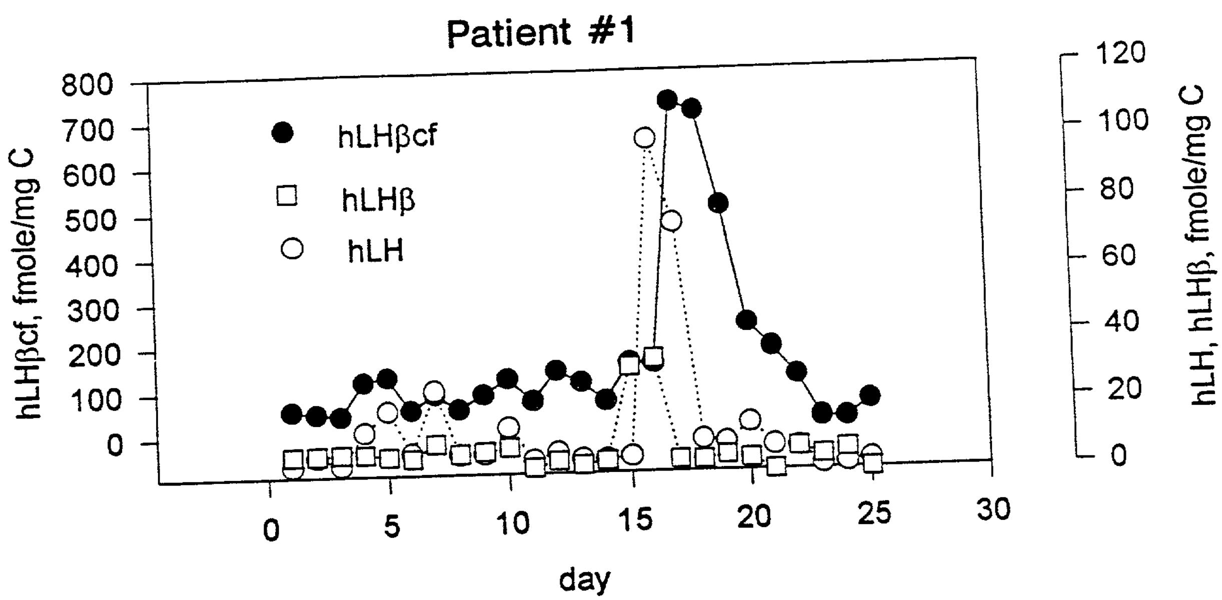


FIGURE 4B

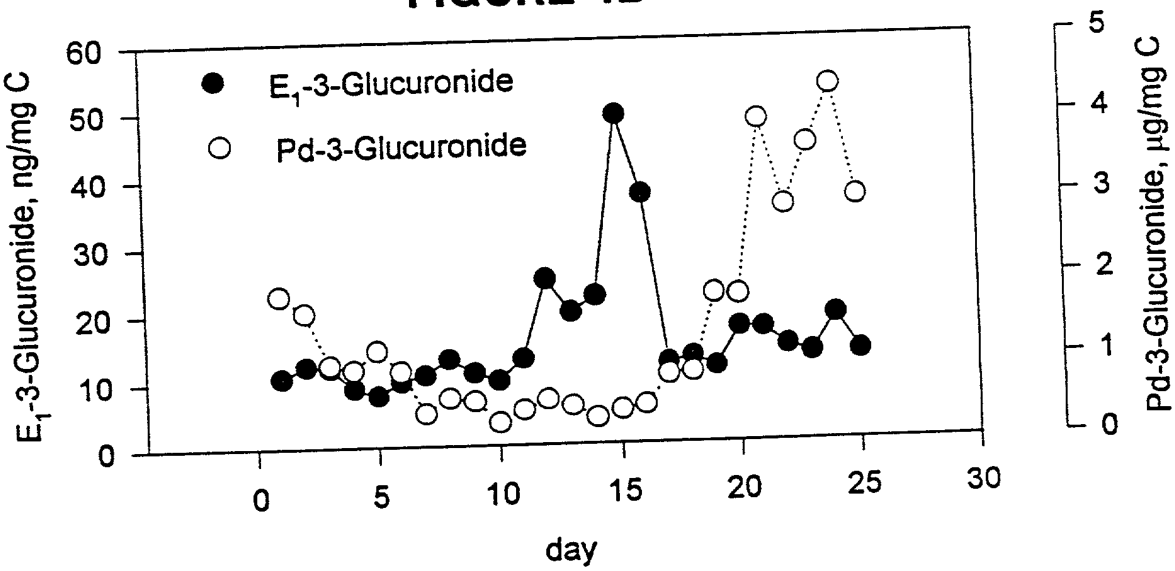


FIGURE 4C

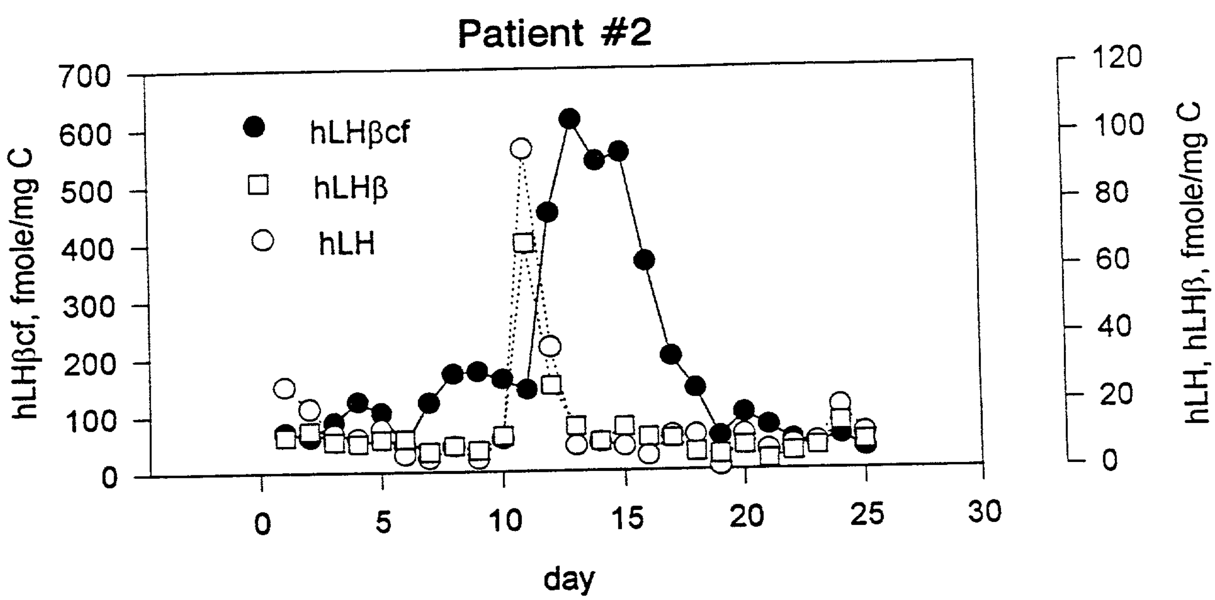


FIGURE 4D

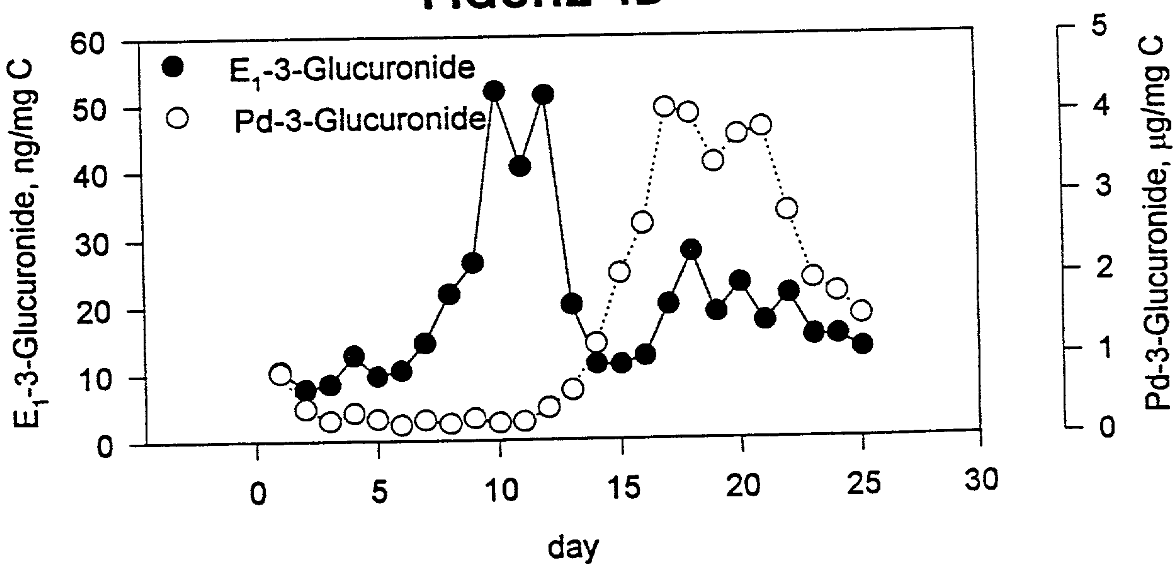


FIGURE 5

HPLC elution positions of the pituitary and urinary hLHbf

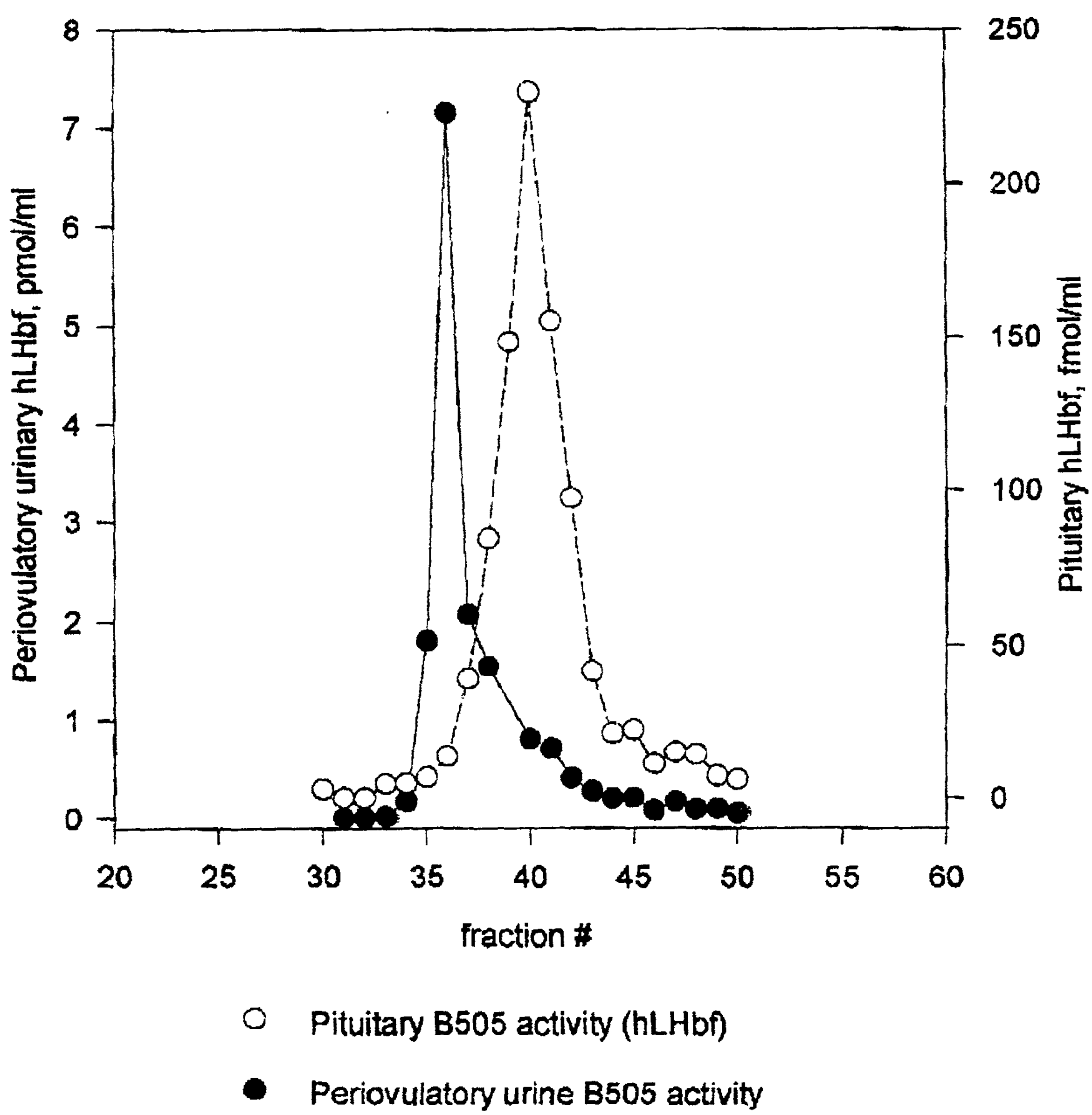


FIGURE 6A

Immunoreactivity testing of pituitary hLHbf after HPLC column

In assay for hLHbf (B505-B503) and in assay for hCG bf (B210-B108)

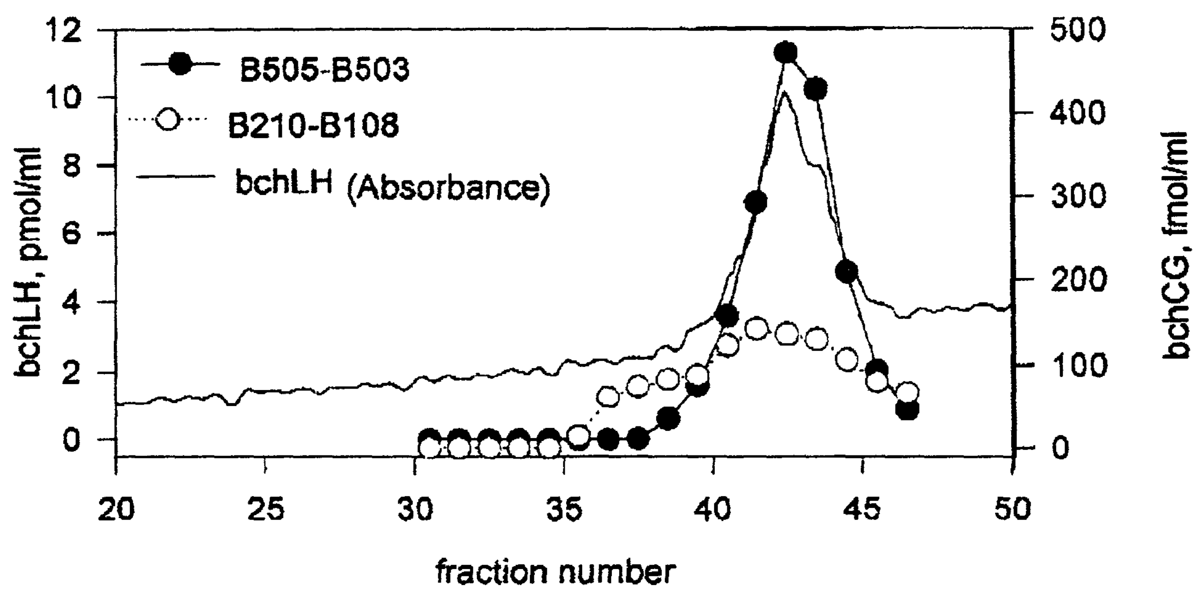


FIGURE 6B

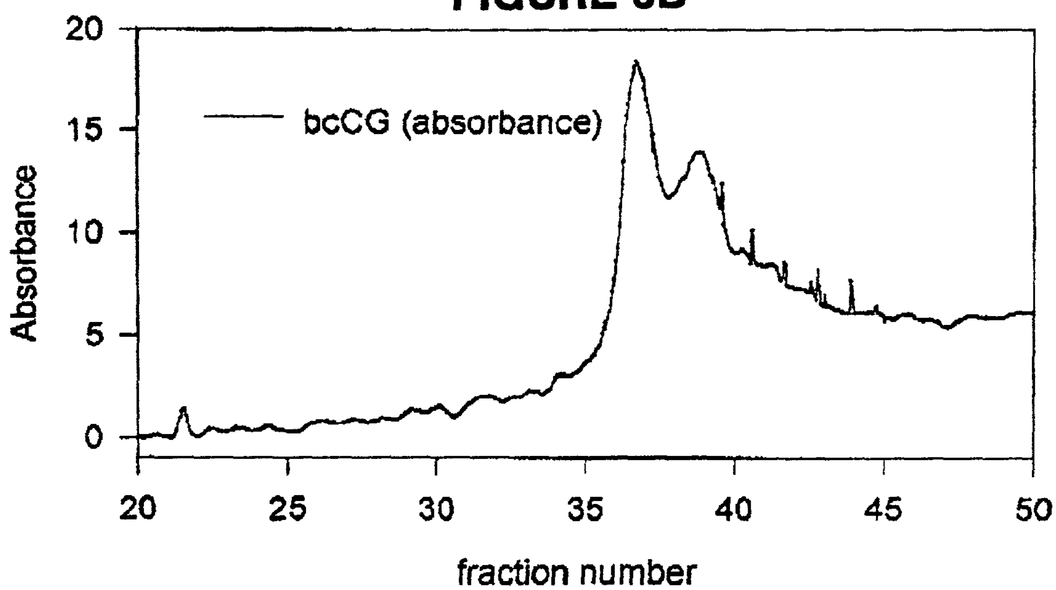
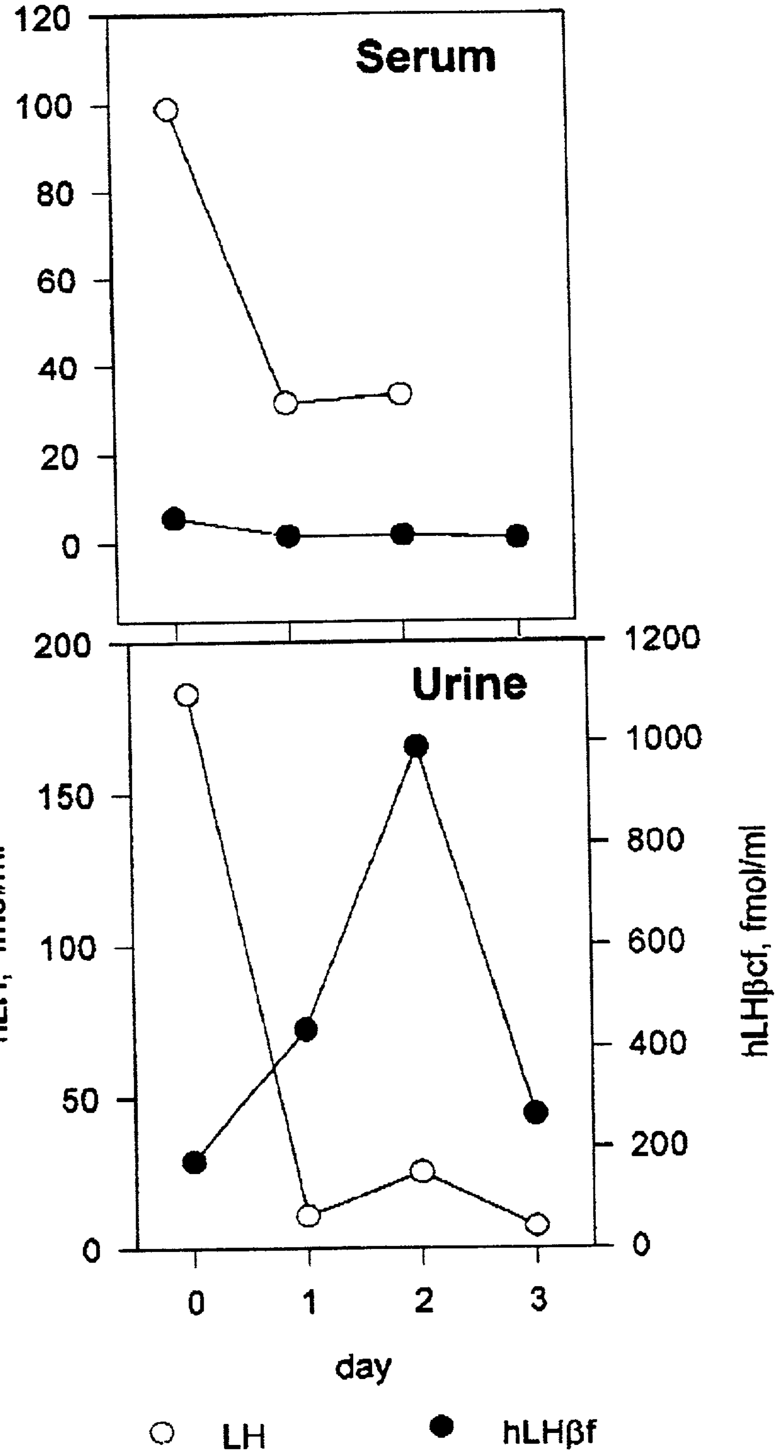


FIGURE 7A

FIGURE 7B



day 0 is the day of LH surge

DECLARATION AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

ANTIBODIES SPECIFIC FOR HLH BETA CORE FRAGMENT AND USES THEREOF

the specification of which (check one)

___ *is attached hereto*

X *was filed on* December 11, 1996 *as*

Application Serial No 08/763669

and was amended ______________ *(if applicable)*

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 (a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International Application which designated at least one country other than the United States, listed below. I have also identified below any foreign application for patent or inventor's certificate, or PCT International Application having a filing date before that of the earliest application from which priority is claimed

Prior Foreign Application(s) *Priority Claimed*

Number	Country	Filing Date	Yes	No
N/A				

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

Provisional Application No.	*Filing Date*	*Status*
60/008,502	December 11, 1995	

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States Application(s), or Section 365(c) of any PCT International Application(s) designating the United States listed below. Insofar as this application discloses and claims subject matter in addition to that disclosed in any such prior Application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56, which became available between the filing date(s) of such prior Application(s) and the national or PCT international filing date of this application:

Application Serial No	*Filing Date*	*Status*
N/A		

And I hereby appoint

John P. White (Reg. No. 28,678); Norman H. Zivin (Reg. No. 25,385); Ivan S. Kavrukov (Reg. No. 25,161); Christopher C. Dunham (Reg. No. 22,031); Robert D. Katz (Reg. No. 30,141); Peter J. Phillips (Reg. No. 29,691); Wendy E. Miller (Reg. No. 35,615); Richard S. Milner (Reg. No. 33,970); Albert Wai-Kit Chan (Reg. No. 36,479); Matthew B. Tropper (Reg. No. 37,457); Robert T. Maldonado (Reg. 38,232); Mary Anne P. Tanner (Reg. No. 40,197); and Mary Catherine DiNunzio (Reg. No. 37,306)

and each of them, all c/o Cooper & Dunham LLP, 1185 Avenue of the Americas, New York, New York 10036, my attorneys, each with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent, to transact all business in the Patent and Trademark Office connected therewith and to file any International Applications which are based thereon under the provisions of the Patent Cooperation Treaty.

Please address all communications, and direct all telephone calls, regarding this application to

John P. White *Reg. No* 28,678
Cooper & Dunham LLP
1185 Avenue of the Americas
New York, New York 10036
Tel. (212) 278-0400

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true: and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first joint inventor Robert E. Canfield

Inventor's signature R. E. Canfield

Citizenship United States of America *Date of signature* 2/25/97

Residence Rockwald, Off Route 301, ½ Mile East of Route 9, Cold Spring, New York 10516 U.S.A.

Post Office Address P.O. Box 220, Cold Spring, New York 10516 U.S.A.

Full name of joint inventor (if any) Steven Birken

Inventor's signature

Citizenship United States of America *Date of signature*

Residence 120 Oneida Avenue, Dumont, New Jersey 07628 U.S.A.

Post Office Address Same As Residing Address

Full name of joint inventor (if any) John O'Connor

Inventor's signature

Citizenship United States of America *Date of signature*

Residence 35 Clinton Place 6A, New Rochelle, New York 10801 U.S.A.

Post Office Address Same As Residing Address

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Inventor's signature ______________________

Citizenship United States of America *Date of signature* ______________________

Residence Rockwald, Off Route 301, ¼ Mile East of Route 9, Cold Spring, New York 10516 U.S.A.

Post Office Address P.O. Box 220, Cold Spring, New York 10516 U.S.A.

Full name of joint inventor (if any) Steven Birken

Inventor's signature Steven Birken

Citizenship United States of America *Date of signature* 2/25/97

Residence 120 Oneida Avenue, Dumont, New Jersey 07628 U.S.A.

Post Office Address Same As Residing Address

Full name of joint inventor (if any) John O'Connor

Inventor's signature ______________________

Citizenship United States of America *Date of signature* ______________________

Residence 35 Clinton Place 6A, New Rochelle, New York 10801 U.S.A.

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Inventor's signature ______

Citizenship United States of America *Date of signature* ______

Residence Rockwald, Off Route 301, ½ Mile East of Route 9, Cold Spring, New York 10516 U.S.A.

Post Office Address P.O. Box 220, Cold Spring, New York 10516 U.S.A.

Full name of joint inventor (if any) Steven Birken

Inventor's signature ______

Citizenship United States of America *Date of signature* ______

Residence 120 Oneida Avenue, Dumont, New Jersey 07628 U.S.A.

Post Office Address Same As Residing Address

Full name of joint inventor (if any) John O'Connor

Inventor's signature John O'Connor

Citizenship United States of America *Date of signature* 2/25/97

Residence 35 Clinton Place 6A, New Rochelle, New York 10801 U.S.A.

Post Office Address Same As Residing Address

Full name of joint inventor (if any) Galina Kovalevskaya

Inventor's signature G. Kovalevskaya

Citizenship Russian *Date of signature* 02/25/97

Residence 850 W. 176th Street 5G. New York. New York 10032 U.S.A.

Post Office Address Same As Residing Address

Full name of joint inventor (if any) __________

Inventor's signature __________

Citizenship __________ *Date of signature* __________

Residence __________

Post Office Address __________

Full name of joint inventor (if any) __________

Inventor's signature __________

Citizenship __________ *Date of signature* __________

Residence __________

Post Office Address __________